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Abstract:
We propose to combine our expertise to target a process that is critical to breast cancer metastasis that is likely conserved in flies, mice and humans. The advantages of addressing the question of metastasis through the combined expertise of the Cagan and Weilbaecher labs is that we will use the powerful genetic tools provided by Drosophila that will identify key genetic pathways critical to tumor cell migration and metastasis that can be rapidly and rigorously tested. This a real time, in vivo dynamic screen that occurs in a whole organism. Tumors develop in the epithelial layer of the wing and the genetics of tumor cell invasion and migration throughout the organism can be modeled in real time, and genetically manipulated in large scale genetic screens. Dr. Weilbaecher's laboratory will take advantage of the genetic knowledge gained from the Drosophila metastasis models in the development of an improved breast cancer metastasis mouse model. Dr. Cagan's laboratory will be provided with mammalian human and murine breast cancers to validate their genetic and pharmacologic anti-metastasis strategies. Jointly, Drs. Cagan and Weilbaecher propose to develop novel therapeutics targeted to the metastatic process. In year one, we have identified 6 compounds that decrease metastasis in Drosophila metastasis model and decrease viability of mammalian breast cancer cells in vitro. We have validated the compound Cyclopamine, a hedgehog inhibitor, to block lung metastases in murine breast cancer xenograft and will use this as a template for testing other candidate therapeutic compounds from fly to mouse. Finally, we have uncovered a previously unknown and important connection between Src and Hedgehog signaling in mediating metastasis.

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INTRODUCTION:

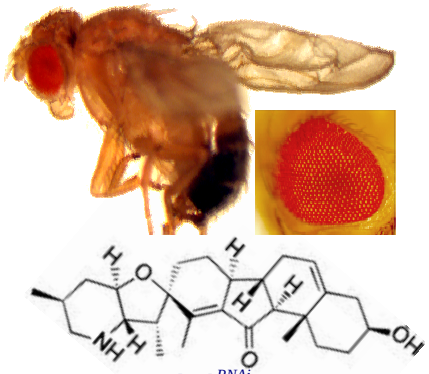
In this Synergy award, we proposed to bring compounds identified with a novel screening method to standard mouse breast cancer assays. We developed Csk/Src Drosophila models to explore specific aspects of overgrowth and metastasis. Both Src and the Csk paralog Chk have been implicated in breast cancer metastasis. We propose to test the hypothesis that drugs identified in our novel Drosophila wing model of tumor (invasive proliferation) and metastasis—targeting the effects of activated Src— will show efficacy in a mouse model of breast cancer and metastasis. The overall goal of this proposal is to validate the findings from a *Drosophila* metastasis model in murine and human breast cancers. Specifically, we will examine the interactions of epithelial tumor cells with bordering non-malignant epithelial cells, and whether these interactions alter the metastatic potential of cells at the tumor boundary. Several critical signaling pathways specific to this interaction have been identified in a *Drosophila* whole animal genetic screen. We propose to validate these pathways in mouse and human breast cancers, and to extend the *Drosophila* search for new factors. The long term goals of this proposal will be to identify critical targets involved in tumor progression and breast cancer metastasis using the power of forward genetics in *Drosophila*, and to develop novel murine breast cancer models of metastasis that can be used to screen new genes and therapeutics targeted to breast cancer metastasis.

BODY:

Our efforts were strongly successful, and we have a joint manuscript under review that presents our work as a new approach to breast cancer therapeutics:

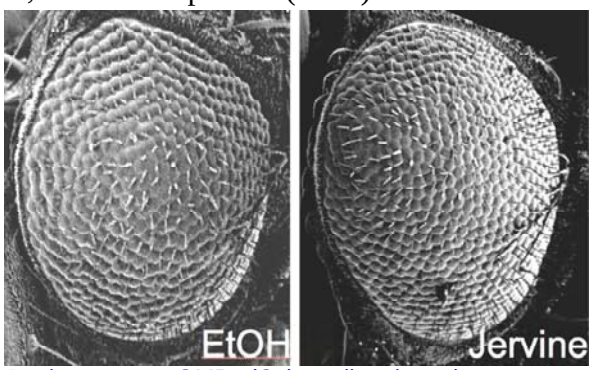
Marcos V., Fink, J., Heller, E., Martinez, E., Salavaggione, L., Weilbaecher, K., and Cagan, R. (2008). Whole Animal Screening for Cancer Therapeutic Compounds Links Src and Hedgehog Activities. *Submitted*.

The Cagan laboratory developed Csk/Src Drosophila models to explore specific aspects of overgrowth and metastasis. Both Src and the Csk paralog Chk have been implicated in breast cancer metastasis. We used these dCsk/Src metastasis models to identify candidate compounds that reduce the oncogenic-like effects of activated Src. Specifically, we utilized a model that contained 'crumpled wing' and 'overgrown eye' phenotypes due to reducing dCsk activity in the eye and wing (*eye/wing>dCsk^{RNAi}*; Figure). The precise genotype was *GMR-GAL4/FM6, MS1096-GAL4 UAS-dCsk^{RNAi}/ MS1096-GAL4 UAS-dCsk^{RNAi}*. We screened the National Cancer Institute "Diversity Set" of 1990 compounds for chemicals that suppressed the *eye/wing>dCsk^{RNAi}* phenotype. The Diversity Set contains an eclectic collection of compounds that emphasize cancer-related compounds. Compounds scored as suppressing the *eye/wing>dCsk^{RNAi}* phenotypes were confirmed in at least two additional re-tests.



Top: *eye/wing>dCsk^{RNAi}*. *Bottom*: Structure of Jervine.

356 compounds (18.2%) permitted animal viability but altered the *GMR>dCsk-IR* phenotype. Of these, 251 drugs had an enhancing effect, and 99 compounds (5.0%) had a suppressive effect. 6 drugs had different effects (suppression *vs.* enhancement) at different concentrations. The 99 compounds that initially suppressed the *GMR>dCsk-IR* phenotype were re-tested in multiple wells. 39 compounds demonstrated consistent phenotypic suppression



Jervine rescues *GMR>dCsk*-mediated rough eye.

Chemical Name	Known functions (incomplete)
2-Hydroxyethyl-3-hydroxypropyl sulfide	
S-(Carboxymethyl)isothiourea	Potential agonist of GABAA and g -aminobutyric acid . Can inhibit Nitrous Oxide synthesis
Ureidosuccinate, *N*-Carbamoyl-*L*-aspartic acid	An intermediate in pyrimidine biosynthesis and, as such, a regulator of cellular metabolism
(4-*tert*-Butyl-2-chlorophenoxy)acetic acid	
2-(2-Chlorobenzyl)succinic acid	Active in a screen against IMP-1 metallo-b-lactamase.
S-Benzoylcysteine	
O-Ethyloxime-octamethylcyclopentanone	
β-Hydroxyhistidine	
Phenacylthioglycolic acid	Efficacy in mouse CD2F1 leukemia model
N-2-Naphthyl-acetoacetamide	Active in suppressing growth in NCI cancer cell line screen
N,N-Dimethyl-*N'*-2-pyridylsulfamide	
2,2-Diphenyl-3-methyl-4-dimethylamino)butyronitrile	Active in suppressing growth in NCI cancer cell line screen
β-Oxo-α-[(2-pyridinylamino)methylene]-cyclopropanepropanenitrile	
Sodium 4-ethoxybenzenediazosul-fonate	
2-Phthalimidoglutaric acid	Thalidomide analog; affects blood vessel density. Some activity in multiple mouse tumor models and on tumor and endothelial cell proliferation.
2-[[(3,4-Dichlorophenyl)methyl]thio]-4,5-dihydro-1H-imidazole	Active in suppressing growth in NCI cancer cell line screen (simple thioureas known to be toxic)
β, β '-dibromo-*N*-methyl-diallylamine	
CAS# [957362-90-2]; NSC 120571	
2-(methylamino)-*N*-[(4-methylphenyl)sulfonyl]-ethanimidoyl chloride	Can regulate metabolism. Active in suppressing growth in NCI cancer cell line screen; imidoyl chloride expected to be reactive
4,4'-(1,2-ethanediyldi-4,1-piperidinediyl)bis[7-chloroquinazoline]	Anti-obesity activity.
Bis(2-amino-4-sulfonamidophenyl)disulfide	Antimicrobial agents. Active in suppressing growth in NCI cancer cell line screen and CDF1 leukemia mouse.

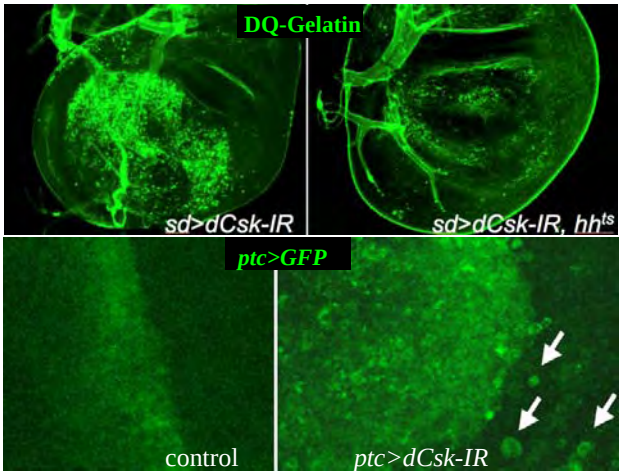
Chemical Name	Known functions (incomplete)
Mitomycin derivative T-53, Aziridinomitosene	DNA crosslinker; effective as an anti-cancer agent; active in suppressing growth in NCI cancer cell line screen and leukemia models
α-[1-(4-Pyridylmethylene)inden-3-yl]-4-pyridinemethanol	Active in suppressing growth in NCI cancer cell line screen
2-(4-Chlorophenyl)-5-methyl-7-(4-methyl-1-piperazinyl)-[1,2,4]triazolo[1,5-α]pyrimidine	Smooth muscle cell growth inhibitors. Active in suppressing growth in NCI cancer cell line screen
3-[(1-Methyl-4-nitro-5-imidazolyl) thio]-5-(3-pyridinyl)-1,2,4-triazole	Active in suppressing growth in NCI cancer cell line screen; known pro-drug functionality releases another thiourea.
N-phenyl-*N'*-(2,2,6,6-tetramethyl-4-piperidinyl)-1,4-benzenediamine	Inhibits Ornithine decarboxylase activity in tumor cells; active in suppressing growth in NCI cancer cell line screen
Mequitazine, LM 209, Virginan, 10-(3-Quinuclidinylmethyl)phenothiazine	Antihistimine; active in suppressing growth in NCI cancer cell line screen; active in mouse CDF1 leukemia models
2-methyl-a-[2-(1-naphthalenylmethyl)phenyl]-benzenemethanimine	Active in suppressing growth in NCI cancer cell line screen
CAS # [903637-76-3]	
Flavazine, Acid Yellow L	
6-(*p*-chlorobenzylthio)-9-(tetrahydropyran-2-yl)-9H-purine	Active in suppressing growth in NCI cancer cell line screen
Blocan, Ampyrox, Restropin	
6-Chloro-7-sulfamoyl-1,1-dioxide-4H-1,2,4-benzothiadiazine-3-propionic acid	Nitric oxide enhancing diuretic compound
2-(1-(4-(2-pyridyl)piperazino))naphthazarin	Identified as toxic in yeast cell cycle mutant strains. Active in suppressing growth in NCI cancer cell line screen.
Jervine	Inhibitor of Hedghog pathway effector Smoothened. Active in suppressing growth in NCI cancer cell line screen.
Mixture of 4,5,6-trichloro-2-methoxy-pyrimidine and 2,4,5-trichloro-6-methoxy-pyrimidine	Active in suppressing growth in NCI cancer cell line screen
2-[(2-Benzothiazolylthio)acetyl]-3-hydroxy-thiazolo[2,3-*b*]benzothiazolium	Can increase cell proliferation at high doses.
(*S*)-4-ethyl-3,4,12,14-tetrahydro-3,14-dioxo-1H-pyrano [3',4':6,7] indolizino[1,2-b]quinolin-4-yl glycine ester monohydrochloride	Pro-drug derivative of camptothecin. Active in suppressing growth in NCI cancer cell line screen and CDF1 leukemia mouse.
Aphidicolin glycinate	DNA polymerase inhibitor; antitumor activity. Active in suppressing growth in NCI cancer cell line screen.

Table . 39 NCI Diversity Set compounds suppressed the *dCsk-IR* phenotype.

(Table below), resulting in a 39.4% repeat rate and 2.0% overall rate of suppressing compounds in the NCI set. This relatively high number of hits likely represents enrichment for cancer-active compounds in the Diversity Set. An example of a hit (Jervine) is provided in the Figure. One compound—2-Phthalimidoglutaric acid— is structurally similar to Thalidomide, and we determined that Thalidomide itself also suppressed the *dCsk-IR* phenotypes as well as proliferation in a mouse breast cancer model (see below).

Jervine is a well-characterized steroidal alkaloid and inhibitor of Hedgehog pathway signaling. Jervine is chemically related to Cyclopamine, and both act through suppression of the Hedgehog receptor Smoothened. The Hedgehog pathway has been recently linked to cancer and, recently, metastasis and has generated significant interest by pharmaceutical companies as a potential cancer target. Jervine consistently though mildly suppressed the *dCsk* phenotype in the eye (Figure above) and wing (not shown). The Hh pathway chemical inhibitor AY9944 also suppressed the *dCsk* phenotype whereas Tomatidine, a Jervine/Cyclopamine-related compound with no Hh activity, did not (Vidal *et al*, *submitted*; not shown).

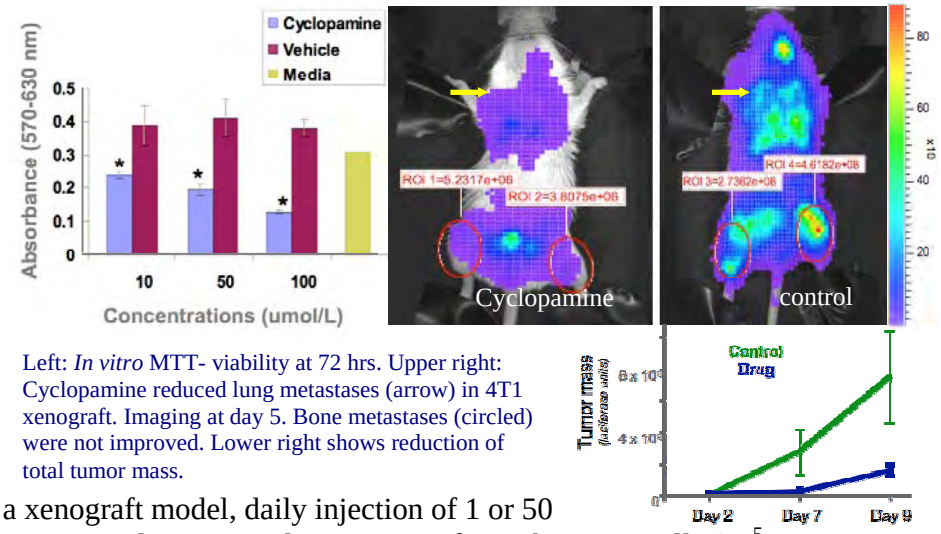
Importantly, genetic mutations that reduced Hedgehog pathway activity led to suppression of *dCsk*-mediated 'metastasis' in our wing and eye models; conversely, mutations in the pathway inhibitor *patched* enhanced migration of cells (not shown). Interestingly, reducing *dCsk* activity in the wing led to expanded expression of the Hh pathway reporter ptc>GFP including within migrating cells, while reducing Hh pathway through *smo* mutations or



Reducing Hh activity (*top*) suppressed the MMP activity reporter DQ-Gelatin and (*bottom*) expanded Hh pathway activity.

through chemical inhibition of Hh signaling suppressed MMP activity (Figure; Vidal *et al, submitted*). Further, reducing Hh pathway activity in the eye mildly but consistently suppressed the *GMR>dCsk-IR* phenotype, while over-expression of the Hh pathway effector Ci enhanced (not shown). These genetic data provide further support for the model that Hedgehog signaling plays an important role in Csk/Src-mediated metastasis. I am not proposing to follow through on the role of *Drosophila* Hedgehog because this is an excellent beginning project for Marcos Vidal as he starts his own laboratory.

Mouse validation: The Weilbaecher laboratory then demonstrated that Jervine and Cyclopamine suppressed expression of the Hedgehog target Gli-1 in both 4T1 breast cancer cells and B16 melanoma cells (not shown) and reduced proliferation of fluorescently-labeled 4T1 breast cancer cells in a cell culture dish (Figure). A similar reduction in proliferation was observed in 5/6 other compounds we tested that were identified from our fly screen. In a xenograft model, daily injection of 1 or 50 mg/kg of Jervine or Cyclopamine consistently prevented metastasis of 4T1-*luc-GFP* cells (10^5 cells injected into the left ventricle of Balb-C mice) to the lung (but not bone); by day 9, tumor mass was reduced an average of more than four-fold (Figure). This data provides important validation that our fly models are able to identify candidate therapeutic compounds that show efficacy in a standard mouse xenograft model.



Left: *In vitro* MTT- viability at 72 hrs. Upper right: Cyclopamine reduced lung metastases (arrow) in 4T1 xenograft. Imaging at day 5. Bone metastases (circled) were not improved. Lower right shows reduction of total tumor mass.

KEY RESEARCH ACCOMPLISHMENTS:

1) Identification of 6 compounds that decrease metastasis in Drosophila metastasis model and decrease viability of mammalian breast cancer cells in vitro.
2) Validation of the compound Cyclopamine, a hedgehog inhibitor, to block lung metastases in murine breast cancer xenograft.
3) This work provides a template for moving candidate therapeutic compounds from fly to mouse.
4) The Drosophila and mouse data demonstrate an important connection between Src and Hedgehog signaling in mediating metastasis.

REPORTABLE OUTCOMES:

This work was presented at the International Cancer and Bone society meeting in Edinburough, Scotland in July 2008. Emanuela Heller, Marcos Vidal, Jill Fink, Lorena Salavaggione, Lourdes Ylagan, Mark Watson, Mark Wilkins, Ross Cagan and Katherine Weilbaecher. Fly to mouse: a new approach to cancer metastasis. *Cancer Treatment Reviews*, 2008, Supplement.

CONCLUSIONS:

We propose to combine our expertise to target a process that is critical to metastasis that is likely conserved in flies, mice and humans. The advantages of addressing the question of metastasis through the combined expertise of the Cagan and Weilbaecher labs is that we will use the powerful genetic tools provided by *Drosophila* that will identify key genetic pathways critical to tumor cell migration and metastasis that can be rapidly and rigorously tested. This a real time, in vivo dynamic screen that occurs in a whole organism. Tumors develop in the epithelial layer of the wing and the genetics of tumor cell invasion and migration throughout the organism can be modeled in real time, and genetically manipulated in large scale genetic screens. Dr. Weilbaecher's laboratory will take advantage of the genetic knowledge gained from the *Drosophila* metastasis models in the development of an improved breast cancer metastasis mouse model. Dr. Cagan's laboratory will be provided with mammalian human and murine breast cancers to validate their genetic and pharmacologic anti-metastasis strategies. Jointly, Drs. Cagan and Weilbaecher propose to develop novel therapeutics targeted to the metastatic process. In year one, we have identified 6 compounds that decrease metastasis in Drosophila metastasis model and decrease viability of mammalian breast cancer cells in vitro. We have validated the compound Cyclopamine, a hedgehog inhibitor, to block lung metastases in murine breast cancer xenograft and will use this as a template for testing other candidate therapeutic compounds from fly to mouse. Finally, we have uncovered a previously unknown and important connection between Src and Hedgehog signaling in mediating metastasis.

REFERENCES:
None

APPENDICES:
None